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(54) Title: ENHANCED EXPRESSION OF VIRAL PROTEINS IN DROSOPHILA CELLS

(57) Abstract

The present invention provides a novel method for enhanced expression of viral proteins, and in particular HIV glycoproteins in *Drosophila* cells.

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Title

ENHANCED EXPRESSION OF VIRAL PROTEINS IN *DROSOPHILA* CELLS

Field of Invention

The present invention relates generally to enhanced expression of viral proteins, and in particular HIV proteins in *Drosophila* cells.

Background of the Invention

Human immunodeficiency virus type 1 (HIV-1) is the etiological agent of acquired immune deficiency syndrome, also known as AIDS. This retrovirus has a complex genetic organization, including the long terminal repeats (LTRs), the *gag*, *pol*, and *env* genes, and other genes. This retrovirus carries a number of viral antigens

which are potential candidates either alone or in concert as vaccinal agents capable of inducing a protective immune response.

Among the more promising of the HIV-1 antigens is the viral envelope glycoprotein (gp160) or specific fragments thereof. The <u>env</u> gene encodes the 160 kilodalton (kd) precursor glycoprotein of the viral envelope. gp160 is cleaved posttranslationally into a 120 kd glycoprotein (gp120) and a 41 kd glycoprotein (gp41), which are present at the virus surface.

gp120, a 481 amino acid glycoprotein, is derived from the amino terminal two-thirds of the gp160 glycoprotein. It is exposed on the outside of the virus, and is crucial to the interaction of the virus with its cellular receptor by binding to the CD4 protein present on the surface of helper T_4 lymphocytes, macrophages, and other cells of the immune system. gp120 thus determines the cellular selectivity of viral infection and contributes to the cytopathogenicity of HIV through its involvement in syncytium formation.

gp41, a 345 amino acid protein derived from the carboxyl terminus of gp160, is an integral membrane protein of HIV-1. gp41 contains a series of hydrophobic amino acids which anchor the protein in the lipid bilayer of the cellular plasma membrane. The carboxyl end of gp41 is believed to protrude into the viral particle. gp41 or a portion thereof is believed to "anchor" gp120 to HIV and is also responsible for fusion between HIV or HIV-infected cells with uninfected cells displaying surface T_4 receptors. The portion of gp41 which is believed to be responsible for this fusion is located at the amino terminus. Such fusion is believed to play a role in viral replication. See, e.g., M. Kowalski et al, <u>Science</u>, <u>237</u>: 1351-55 (1987); D.M. Knight et al, <u>Science</u>, <u>236</u>: 837-36

(1987).

These viral glycoproteins assume a tertiary structure as viral spikes protruding outwards from the surface of the viral particle. About 70 to 80 spikes are believed to be associated with each newly synthesized viral particle. As the viral particle ages, the spikes disappear, apparently because the association between the gp120 and gp41 is weak. Thus, for newly synthesized viral particles, this viral glycoprotein spike is believed to be the most immediate target accessible to the immune system following infection.

Virus neutralizing antibodies have been reported directed against gp120 and gp41 epitopes. It has been specifically noted that a target site for type specific neutralizing antibodies is located in the 3' half of the gp120 glycoprotein molecule.

The _env_ gene of HIV-1 has thus been the target of numerous recent investigations. Expression of glycosylated gp160 has previously been obtained in mammalian cells and certain baculovirus insect cells by groups which have also reported the induction of both humoral and cellular immune responses to these antigens. gp120 has been expressed recombinantly with the use of heterologous promoters in several systems. See, e.g., S. Chakrabarti et al, _Nature_ (London), _320_: 535 (1986); S.I. Hu et al, _Nature_ (London), _320_: 537 (1986); and M.P. Kieny et al, _Biotechnology_, _4_: 790 (1986).

L.A. Lasky et al, _Science_, _233_: 209-212 (1986) constructed a number of plasmids containing mutant _env_ genes for tranfection into mammalian cells, specifically Chinese hamster ovary (CHO) cells. Lasky et al. report secretion of a gene product encoded by a plasmid containing the first 50 amino acids of the glycoprotein D (gD) protein joined in phase to an amino acid sequence comprising (#61-#531) of the HIV _env_ protein. A

recombinant envelope antigen was produced containing 25 amino acids of gD at its amino terminus. The resulting gene was 520 amino acids in length.

Knight et al, cited above, describe expression of the art/trs transactivator protein of HIV in mammalian cells. The mammalian cell line used for expression of these HIV proteins was the COS-7 monkey cell line. These plasmids utilized the HIV LTR as a promoter and RNA processing signals from SV40 to express the inserted DNA as a functional messenger RNA. To express gp120, a plasmid pENV160 was developed which contains the entire coding region of the env gene fused to the HIV LTR.

U.S. Patent 4,725,669 also discloses glycoproteins of approximately 160 kd and 120 kd obtained from the human H9/HTLV-III cell line, each having an approximately 90 kd unglycosylated moiety.

D.L. Lynn, et al, in "Mechanisms of Control of Gene Expression", Eds. Allan R. Liss Inc., pp. 359-368 (1988) disclose the cloning of the entire gp160 gene behind the polyhedron promoter of the baculovirus Autographa californica. Spodoptera cells infected with the recombinant virus express a protein that is released from the cell upon lysis.

The HIV-1 virus also encodes two regulatory proteins, tat and rev, which govern viral gene expression and which are essential for virus replication. The tat protein increases the expression of both structural and regulatory proteins of HIV while the rev protein selectively increases the synthesis of structural proteins.

The precise mechanism of rev function remains unknown. It is known that rev is primarily localized in the nucleolus. This localization is thought to be important for rev function. Hence it is thought that rev regulates gene expression by facilitating export of the nuclear-entrapped mRNA into the cytoplasm. Rev has also

been shown to function in a number of different mammalian cell types, e.g., human, monkey and hamster. However, rev regulation has not been demostrated in any non-mammalian system.

Therefore it is an object of the present invention to express rev in Drosophila. It is a further object of the present invention to enhance the production of viral proteins in Drosophila using rev.

Summary of the Invention

In one aspect, the present invention is an HIV rev expression unit which includes a DNA coding sequence and regulatory sequences necessary for transcription of the rev protein coding sequence and subsequent translation within a Drosophila cell.

In related aspects, this invention is a DNA vector which comprises the gene expression unit of the present invention.

In yet another related aspect, this invention is a Drosophila cell transfected with the DNA vector of this invention.

In further related aspects, this invention is an HIV rev protein, or a derivative thereof produced by the transfected cells of this invention. The derivative encompasses any rev protein such as deletions, additions, substitutions or rearrangement of amino acids or chemical modifications thereof which retain the ability to be recognized by antibodies raised to the wild-type rev protein.

This invention also relates to a method for enhancing the production of viral proteins in insect cells. The method entails culturing Drosophila cells

transfected with a gene expression unit for a viral protein of interest and a rev expression unit in a suitable medium such that the transfected cells are capable of expressing the protein of interest. The protein may thereafter be collected from the cell or cell culture medium.

In another aspect, this invention is a whole cell vaccine for stimulating protection against HIV infection, which comprises an immunoprotective and non-toxic quantity of an HIV protein associated with an inactivated Drosophila cell.

This invention further relates to a method for protecting a human against disease symptoms associated with HIV infection, which comprises administering to a human a safe and effective amount of the whole cell vaccine of the present invention.

Detailed Description of the Invention

The method and expression system of the present invention facilitate high-level production of viral proteins, particularly HIV env proteins and derivatives thereof, in a Drosophila cell culture. The Drosophila cells are transfected by using standard techniques which permit introduction of foreign DNA into a host cell without adversely affecting the foreign DNA or the host cell. The recombinant Drosophila cells so constructed produce viral proteins.

One feature of the present invention is the enhanced expression of viral structural proteins (e.g., env, pol, and gag) when coexpressed with the rev protein in Drosophila. In contrast to the tat protein which functions poorly, if at all, the rev protein appears to be fully functional when produced by the present invention.

For example, the HIV-1 env protein, gp160, is barely expressed in the absence of rev. Upon coexpression with rev in Drosophila, the levels of gp160 are enhanced (5 to 10 fold).

Analysis of total RNA demonstrated that synthesis of gp160 message was dependent on induction of the Drosophila Mt promoter and was independent of Rev. However, an analysis of fractionated RNA revealed that full-length, unspliced gp160 mRNA was found in the cytoplasm only in the presence of Rev. In the absence of Rev, this RNA was apparently retained in the nucleus.

In contrast to the Baculovirus system of the prior art in which the HIV protein is provided only upon lysis of the infected insect cells, the method of this invention provides a continuous cell expression system for HIV proteins.

The protein of the present invention may be secreted, and purification from the culture medium is by conventional techniques. Alternatively, the protein of the present invention may be produced intracellularly or membrane-bound, and the protein may be extracted from the cells using conventional techniques. Alternatively, membrane-bound protein may be employed in a variety of cell-associated assays, or used as a whole-cell vaccine.

A preferred Drosophila cell line for use in the practice of the invention is the D. melanogaster S_2 line. S_2 cells [Schneider, J. Embryol. Exp. Morph. 27: 353 (1972)] are stable cell cultures of polyploid embryonic Drosophila cells. Introduction of the DNA coding sequence for gp120, or derivatives thereof, into Drosophila S_2 cells by DNA transfection techniques produces unexpectedly large amounts of the glycoprotein. Use of the S_2 Drosophila cell has many advantages, including, but not limited to, its ability to grow to a high density at room temperature. Stable integration of

the selection system has produced up to 1000 copies of the transfected gene expression unit into the cell chromosomes.

Other Drosophila cell culture systems may also be useful in the present invention. Some possibly useful cells are, for example, the KC-O Drosophila Melanogaster cell line which is a serum-free cell line [Schulz et al, Proc. Nat'l Acad. Sci. USA, 83: 9428 (1986)]. Preliminary studies using the KC-O line have suggested that transfection is more difficult than with S_2 cells. Another cell line which may be useful is a cell line from Drosophila hydei. Protein expression can be obtained using the hydei cell line; however, transfection into this cell line can result in the transfected DNA being expressed with very low efficiency [Sinclair et al, Mol. Cell. Biol., 5: 3208 (1985)]. Other available Drosophila cell lines which may be used in this invention include S_1 and S_3.

The Drosophila cells selected for use in the present invention can be cultured in a variety of suitable culture media, including, e.g., M_3 medium. The M_3 medium consists of a formulation of balanced salts and essential amino acids at a pH of 6.6. Preparation of the media is substantially as described by Lindquist, DIS, 58: 163 (1982). Other conventional media for growth of Drosophila cells may also be used.

A recombinant DNA molecule or vector containing a viral protein gene expression unit can be used to transfect the selected Drosophila cells, according to the invention. The gene expression unit contains a DNA coding sequence for a selected viral protein or for a derivative thereof. Such derivatives may be obtained by manipulation of the gene sequence using traditional genetic engineering techniques, e.g., mutagenesis, restriction endonuclease treatment, ligation of other gene sequences including synthetic sequences and the like. See, e.g., T. Maniatis

et al, Molecular Cloning, A Laboratory Manual., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).

The HIV DNA coding sequence, which includes rev, has been published. See, Ratner et al, Nature 313:277-284 (1985) or Wain-Hobson et al, Cell 40:9-17 (1985). The nucleotide sequence is also available from GenBank (clone BH10, Ratner et al, supra).

DNA molecules comprising the coding sequence of this invention can be derived from HTLV-III infected cells using known techniques (see, Hahn et al, Nature 312:166-169 (1984)), or, in the alternative, can be synthesized by standard oligonucleotide techniques, or via PCR. Moreover, there are numerous recombinant host cells containing the cloned DNA coding sequences, which are widely available.

Derivatives can then be prepared by standard techniques, including DNA synthesis. Such derivatives may include, e.g., rev, gp120 or gp160 molecules in which one or more amino acids have been substituted, added or deleted without significantly adversely affecting the binding capacity or biological characteristics of the protein. Derivatives of these proteins may also be prepared by standard chemical modification techniques, e.g., acylation, methylation.

Also included in the gene expression unit are regulatory regions necessary or desirable for transcription of the protein coding sequence and its subsequent translation and expression in the host cell. The regulatory region typically contains a promoter region which functions in the binding of RNA polymerase and in the initiation of RNA transcription. The promoter region is found upstream from the protein coding sequence.

Preferred promoters are of Drosophila origin, e.g., the Drosophila metallothionein promoter [Lastowski-Perry et al, J. Biol. Chem., 260: 1527

(1985)]. This inducible promoter directs high-level transcription of the gene in the presence of metals, e.g., $CuSO_4$. Use of the Drosophila metallothionein promoter results in the expression system of the invention retaining full regulation even at very high copy number. This is in direct contrast to the use of the mammalian metallothionein promoter in mammalian cells in which the regulatory effect of the metal is diminished as copy number increases. In the Drosophila expression system, this retained inducibility effect increases expression of the gene product in the Drosophila cell at high copy number.

The Drosophila actin 5C gene promoter [B.J. Bond et al, Mol. Cell. Biol., 6: 2080 (1986)] is also a desirable promoter sequence. The actin 5C promoter is a constitutive promoter and does not require addition of metal. Therefore, it is better-suited for use in a large scale production system, like a perfusion system, than is the Drosophila metallothionein promoter. An additional advantage is that the absence of a high concentration of copper in the media maintains the cells in a healthier state for longer periods of time.

Examples of other known Drosophila promoters include, e.g., the inducible heatshock (Hsp70), the COPIA LTR, and the α-tubulin promoters. The SV40 early promoter gives lower levels of expression than the Drosophila metallothionein promoter. Promoters which are commonly employed in the mammalian cell expression vectors including, e.g., avian Rous sarcoma virus LTR and simian virus (SV40 early promoter) demonstrate poor function and expression in the Drosophila system.

A gene expression unit or expression vector for the viral protein of interest may also be constructed by fusing the viral protein coding sequence to a desirable signal sequence. The signal sequence functions to direct

secretion of the protein from the host cell. Such a signal sequence may be derived from the sequence of tissue plasminogen activator (tPA). Other available signal sequences include, e.g., those derived from Herpes Simplex virus gene HSV-I gD [Lasky et al, Science, supra.].

The DNA coding sequence for the protein of interest may also be followed by a polyadenylation (poly A) region, such as an SV40 early, or SV40 late, or metallothionein poly A region. The poly A region which functions in the polyadenylation of RNA transcripts appears to play a role in stabilizing transcription. A similar poly A region can be derived from a variety of genes in which it is naturally present. This region can also be modified to alter its sequence provided that polyadenylation and transcript stabilization functions are not significantly adversely affected.

The recombinant DNA molecule may also carry a genetic selection marker, as well as the viral protein gene. The selection marker can be any gene or genes which cause a readily detectable phenotypic change in a transfected host cell. Such phenotypic change can be, for example, drug resistance, such as the gene for hygromycin B resistance.

Alternatively, a selection system using the drug methotrexate, and prokaryotic dihydrofolate reductase (DHFR) gene, can be used with Drosophila cells. The endogenous eukaryotic DHFR of the cells is inhibited by methotrexate. Therefore, by transfecting the cells with a plasmid containing the prokaryotic DHFR which is insensitive to methotrexate and selecting with methotrexate, only cells transfected with and expressing the prokaryotic DHFR will survive. Unlike selection of transformed mammalian and bacterial cells, in the Drosophila system, methotrexate can be used to achieve initially high-copy number transfectants. Only cells

which have incorporated the protective prokaryotic DHFR gene will survive. Concomitantly, these cells have the gene expression unit of interest.

Once a recombinant DNA molecule or expression vector containing the viral protein gene expression unit and the _rev_ gene expression unit has been constructed, it can be transfected into the _Drosophila_ cell using standard transfection techniques. Such techniques are known to those of skill in the art and include, for example, calcium phosphate co-precipitation, cell fusion, electroporation, microinjection and viral transfection.

A one, two, or three vector system can be used in the present invention to transfect a _Drosophila_ host cell. For example, in a three vector system, the gene expression unit for the desired protein (e.g., an HIV _env_ protein or derivative) and the _rev_ expression unit and the coding region for a selectable marker are all located on different vectors. It is noted that all three elements, the desired protein expression unit, the _rev_ expression unit, and the selectable marker can also be found on one or two vectors. A preferred illustrative embodiment of this invention is the production of an HIV _env_ protein employing a vector containing an HIV protein expression unit, e.g., pgp160Δ32, a vector containing the _rev_ expression unit, e.g., pMtRev, and a vector containing the hygromycin B gene expression unit, e.g., pCOHYGRO.

pgp160Δ32 contains an expression unit comprising the _Drosophila_ metallothionein promoter, a derivative of the gp160 gene, and the SV40 poly A site. This gp160 expression unit in combination with _rev_ and the pCOHYGRO vector system will produce a gp160 derivative in S_2 _Drosophila_ cells. Moreover, the antibiotic hygromycin B can be used to select for those cells containing the transfected vectors. A more complete description of this embodiment is described in Example 2.

As another example, an expression system employing the DHFR gene/methotrexate selection system, consisting of the vectors pgp160Δ32, pMtRev and pHGCO, can be used to select methotrexate-resistant cells expressing gp160 or a derivative thereof. The pHGCO vector comprises a DHFR gene expression unit and is co-transfected with pgp160Δ32 and pMtRev, thereby providing the DHFR gene necessary for selection. These selectable markers are further described by Johansen et al, U.S. Patent Application Serial No. 07/047,736, filed May 8, 1987 and is incorporated by reference herein.

According to the invention, the vectors are transfected into <u>Drosophila</u> S_2 cells using conventional techniques. Vectors containing the protein expression unit of interest (e.g., HIV gp160) and the <u>rev</u> expression unit are preferably present in the same molar ratios. The vector having the coding sequence for the selectable marker may be added in varying ratios depending upon the particular copy number of the gene of interest desired. The transfected cells are then selected, such as in M_3 medium containing serum and the appropriate selection agent, e.g., hygromycin B or methotrexate.

Once an appropriate vector has been constructed and transfected into the selected <u>Drosophila</u> cell line, the expression of gp160 is induced by the addition of an appropriate inducing agent for the inducible promoter. For example, cadmium or copper are inducing agents for the metallothionein promoter. Heat is the inducing agent for the Hsp70 promoter. For constitutive promoters, such as the actin 5C promoter, no inducing agent is required for expression.

Transcription and expression of the viral protein coding sequence in the above-described systems can be monitored. For example, Southern blot analysis can be used to determine copy number of the gp160 gene. Northern

blot analysis provides information regarding the size of the transcribed gene sequence [see, e.g., Maniatis et al, cited above]. The level of transcription can also be quantitated. Expression of the selected HIV protein in the recombinant cells can be further verified through Western blot analysis and activity tests on the resulting glycoprotein.

Drosophila S_2 cells are especially suited to high-yield production of protein in the method of the present invention. The cells can be maintained in suspension cultures at room temperature (24±1°C). Culture medium is M_3 supplemented with between 5 and 10% (v/v) heat-inactivated fetal bovine serum (FBS). In the preferred embodiment of the invention, the culture medium contains 5% FBS. After induction, the cells may be cultured in serum-free media. When the pCOHYGRO vector system is used, the media is also supplemented with 300 µg/ml hygromycin B. In this media, the S_2 cells can be grown in suspension cultures, for example, in 250 ml to 2000 ml spinner flasks, with stirring at 50-60 rpm. Cell densities are typically maintained between 10^6 and 10^7 cells per ml. In one embodiment of this invention, the cells are grown prior to induction in 1500 ml spinner flasks in media containing 5% serum.

Following cell culture, the viral protein can be isolated from the spent media, e.g., by use of a monoclonal antibody affinity column. Other known protein purification steps, e.g., metal chelates, various affinity chromatography steps or absorption chromatography, can be used to purify the viral protein from the culture media. The glycoproteins produced by Drosophila cells, according to this invention, are completely free of contaminating materials, e.g., mammalian, yeast, bacterial and more importantly, other (HIV) viral materials. Drosophila-produced HIV proteins have also been

demonstrated to possess different pattern of glycosylation than that reported by other systems, e.g., mammalian systems.

The HIV proteins and derivatives produced, according to the present invention, may be useful in a variety of products. For example, these recombinant proteins may be used in pharmaceutical compositions for the treatment of HIV-infected subjects. Such a pharmaceutical composition, according to the present invention, comprises a therapeutically effective amount of the HIV protein or derivative of the invention in admixture with a pharmaceutically acceptable carrier. The composition can be systemically administered either parenterally, intravenously or subcutaneously. When systemically administered, the therapeutic composition for use in this invention is in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such a parenterally acceptable protein solution, having due regard to pH, isotonicity, stability and the like, is within the skill of the art.

The dosage regimen will be determined by the attending physician, considering various factors which modify the action of drugs, e.g., the condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. The pharmaceutical carrier and other components of a pharmaceutical formulation would be selected by one of skill in the art.

Additionally, the recombinant proteins of the present invention may be used as whole cell vaccines to innoculate mammalian subjects against HIV infection. The cells may be inactivated by physical (e.g., heat) or chemical means (e.g. addition of glutaraldehyde). The preparation of vaccines is generally described in Voller et al. (eds.), <u>New Trends and Developments in Vaccines</u>,

University Park Press, Baltimore, Maryland (1978).

The following examples illustrate the construction and transfection of exemplary vectors of the present invention. These examples are not to be considered as limiting the scope of this invention.

Restriction enzymes and other reagents were used substantially in accordance with the vendors' instructions.

Examples

Example 1. Vector Constructions

a) pMTtPA

As the basic vector for gene expression in *Drosophila*, the tPA expression vector pMTtPA (also called pDMtPA) was used. This vector is a derivative of vector pML1, a small pBR322 vector containing the beta-lactamase gene which has the poison sequences [Mellon et al, *Cell*, *27*: 297 (1982)] deleted from it. These sequences are inhibitory to amplification of the vector. This vector was digested with *Sal*I and *Aat*II which removes a small piece of pBR322, and the digested ends were filled in. The missing piece of pBR322 was then replaced with a cassette containing the *Drosophila* metallothionein promoter on an end-filled *EcoR1*-*Stul* fragment, followed by a filled-in *Hind*III-*Sac*1 fragment from pDSPI [D.S. Pfarr et al, *DNA*, *4*(6): 461 (1985)] containing a tPA sequence containing the signal sequence, prepeptide and the entire coding region of tPA. The tPA gene on this fragment is followed by an SV40 early polyadenylation site.

b) pgp160Δ32

A HindIII-XbaI fragment containing the entire env gene was isolated from an HIV-isolate clone BH10 [L. Ratner et al, Nature, 313:277-84 (1985); see also GenBank]. The entire gp160 sequence was then inserted into a NcoI-XbaI digested vector pDSP1. The resulting vector, SU2, was digested with NdeI, followed by treatment with mung bean nuclease and subsequently digested with SacI, thus isolating the gp160 gene. The digestion with NdeI cut the gp160 sequence at amino acid #32. The SacI digestion cuts 3' of the gp160 gene, including part of the original pDSP1 vector containing a polylinker. This fragment was inserted into the above-described expression vector pMTtPA which had been digested with BglII, end-filled, and subsequently cut with SacI, which deletes the mature tPA sequence. This creates a coding sequence for the first 36 amino acids of tPA (i.e., signal sequence) fused to 795 amino acids of gp160 beginning with amino acid number 32 (asp) of the mature viral molecule and ending at the natural gp160 stop codon.

c) pgp120FΔ32

Another vector containing a modified gene sequence was constructed by digesting pgp160Δ32 with HindIII and SacI, thereby removing the carboxyl terminus of gp160. Approximately two-thirds of the sequence coding for gp41 is removed by this digestion. Thus, this gp160 sequence is missing the first 31 amino acids and the last 216 amino acids of the natural gp160 sequence. The deleted sequence at the carboxy terminus was replaced by a short synthetic DNA linker encoding a stop codon on an HindIII-SacI fragment. The 6-amino-acid linker sequence is as follows:

5'AGCTTTGACTGACTGAGCT 3'.

d) pgp120Δ32

Yet another vector containing a mutant gp160 gene was constructed by digesting pgp160Δ32 with StyI and XbaI, thereby deleting all of the sequence for gp41 and about 30 amino acids at the carboxyl terminus of the gp120 glycoprotein sequence. This fragment was replaced by a synthetic StyI-XbaI linker sequence coding for the correct carboxyl terminus of gp120 from the StyI site to the processing site of gp120-gp41. This sequence was followed by a stop codon. This sequence thereby contained all of the coding sequence for gp120 minus the first 31 amino acids and none of the gp41 coding sequence.

e) pgp120Δ274

Still another exemplary vector containing a mutant gp120 gene was constructed as follows: a 720-base pair carboxyl terminal fragment of gp120 was isolated by a partial digestion of pgp120Δ32 with BglII followed by a XbaI digestion. This fragment was now inserted in place of the tPA gene into the BglII-XbaI cut pMTtPA expression vector. The resulting vector, p120Δ274, contains a coding sequence for the first 36 amino acids of tPA (i.e., the signal sequence) fused to amino acid number 275 of the mature gp120 molecule.

f) pgp160Δ0

An ApaLI-SacI fragment was isolated from plasmid pgp160Δ32 containing the majority of the gp160 coding sequence. A BglII-ApaLI fragment encoding the N-terminus of the mature gp160 coding sequence was generated by the

PCR technique using the natural gp160 coding sequence from the BH10 clone (see (b)) as the template. (The BglII site was introduced at the first codon of mature gp160). This BglII-ApaLI fragment and the ApaLI-SacI fragment were used to replace the pgp160Δ32 coding sequence which was removed by digestion with BglII-SacI. The resulting vector encodes the entire mature gp160 coding sequence and contains all of the regulatory elements as found in gp160Δ32.

g) pMtRev

The entire tPA coding sequence (i.e., for the signal sequence and mature protein) of pMTtPA is replaced with a polylinker region. This plasmid is herein referred to as pMtpolyA. pMtRev is then contructed by inserting an XbaI-XhoI fragment encompassing rev cDNA isolated from plasmid pH3art (Rosen et al., Proc Nat'l Acad Sci USA, 85:2071-6 (1988)) into the XbaI-XhoI sites of the polylinker region of pMtpolyA. The resulting vector encodes the Drosophila metallothionein promoter, the rev protein, and the SV40 polyA region.

h) pCOHYGRO

A commercially available plasmid, pUC18 [BRL] containing a BamHI and SmaI site was used. The 5' LTR from an integrated COPIA element (357 base pairs) was cloned into the BamHI site of vector pUC18, resulting in the vector designated pUCOPIA. COPIA is a representative member of the disperse middle repetition sequences found scattered through the Drosophila genome [Rubin et al, in Cold Spring Harbor Symp. Quant. Biol., 45: 619 (1980)]. The vector pUCOPIA was cut at the SmaI site and the E. coli gene coding for hygromycin B phosphotransferase

(hygromycin B cassette) was cloned into pUCOPIA using standard cloning techniques. The hygromycin B cassette was isolated on a HindIII-BamHI fragment of 1481 base pairs from the vector DSP-hygro [Gertz et al, Gene, 25: 179 (1983)]. The hygromycin B cassette contains the sequence coding for the hygromycin B phosphotransferase gene and the SV40 early poly A region. The HindIII and BamHI sites were filled in using T_4 DNA polymerase, and the hygromycin B cassette was ligated into the SmaI site of the vector pUCOPIA producing vector pCOHYGRO.

Example 2. Transfection into Drosophila S_2 Cells

pCOHYGRO was transfected into S_2 Drosophila cells together with a vector carrying a gp160 mutant gene (e.g., pgp160Δ32) and the rev gene, both of which were under the control of the Drosophila metallothionein promoter as described above. A total of 20 μg of plasmid DNA was used in each transfection which consisted of 10 μg of the hygromycin B selection plasmic pCOHYGRO and 10 μg total of pMt160Δ32 and pMtRev. The transfected cells were selected in M_3 medium containing 10% serum and 300 μg/ml of hygromycin B. After 2 to 3 days under identical conditions, the untransfected cells stop dividing and begin to die. The time of selection in order to obtain stable, growing hygromycin B-resistant cells in the transfected cultures is approximately two to three weeks. Expression of the pgp160Δ32 gene product was verified after induction of the metallothionein promoter with 500 μM $CuSO_4$. Expression of gp160 was observed when rev protein was supplied in trans.

When the transfection was done in the absence of rev, there was very little, if any, protein observed. Northern blot analysis of total RNA revealed that a full length transcript was efficiently produced upon induction,

however, the transcript was retained in the nucleus and could not be detected in the cytoplasm. When a rev expression vector was transfected with the pgp160Δ32 vector, a gp160 protein was observed on a Western blot where no protein was observed before. Furthermore, gp160 production increased with increasing levels of rev protein in the cells. Analysis of the RNA indicated that gp160 mRNA was now abundanat in the cytoplasm. Hence, this is the first demonstration of rev regulation which functions in in a non-mammalian cell type. In addition, the expression of gp120 from gp120Δ32 is Rev-independent.

It was further observed that the protein encoded by gp160Δ32 appears to be cleaved to produce a gp120-sized molecule. This gp120-sized molecule rapidly dissociates from the cell and is found in the culture medium. This gp120 protein also recognizes and binds to a soluble form of the human CD4 protein and thus retains at least its receptor recognition properties. The dissociation of the Drosophila expressed gp120 molecule appears to be due to the fact that it is lacking the N-terminal 31 amino acids of the mature viral protein. Expression from an otherwise identical gp160 construct (i.e., pgp160Δ0) in which these 31 amino acids have been restored produces gp120 which remains associated with the cells.

The above description and examples fully disclose the invention, including preferred embodiments thereof. Modifications of the methods described, e.g, employing other viral proteins or truncated gp160 sequences that are obvious to one of ordinary skill in the art of molecular genetics and related sciences, are intended to fall within the scope of the following claims.

What is claimed is:

1. An HIV _rev_ gene expression unit comprising a DNA coding sequence for said protein and a regulatory element necessary for the transcription of the coding sequence and translation within a _Drosophila_ cell.

2. The gene expression unit of claim 1 wherein the regulatory element is of _Drosophila_ origin.

3. The gene expresion unit of claim 2 wherein the regulatory element comprises an actin 5C promoter, metallothionein promoter, Hsp-70 promoter, α-tubulin promoter or the 5'LTR of a copia element.

4. The gene expresion unit of claim 1 wherein the regulatory element comprises the _Drosophila_ metallothionein promoter.

5. A DNA vector comprising the _rev_ gene expression unit of claim 1.

6. A _Drosophila_ cell transfected with the vector of claim 5.

7. An HIV _rev_ protein produced in a culture of insect cells as found in claim 1.

8. A method for enhancing production of viral proteins in insect cells which comprises culturing in a suitable medium _Drosophila_ cells cotransfected with an viral gene expression unit and a _rev_ gene expression unit, said cells being capable of expressing said viral protein and _rev_.

9. The method of claim 8 wherein the viral protein is an HIV protein.

10. The method of claim 9 wherein the HIV protein is an HIV _env_ protein.

11. The method of claim 10 wherein the HIV _env_ protein is gp160.

12. The method of claim 9 wherein the HIV protein is _gag_.

13. The method of claim 9 wherein the HIV protein is pol.

14. The method of claim 8 wherein the ratio of the viral gene expression unit and the rev gene expression unit is 1:1.

15. The method of claim 8 wherein said cells are transfected with an additional vector containing the coding sequence for the hygromycin B phosphotransferase gene expression unit.

16. The method of claim 15 wherein the hygromycin B phosphotransferase gene expression unit is found on pCOHYGRO.

17. A whole cell vaccine for stimulating protection against HIV infection wherein such vaccine comprises an immunoprotective and non-toxic quantity of an HIV protein associated with an inactivated Drosophila host cell.

18. A method for protecting a human against disease symptoms associated with HIV infection which comprises administering to such human a safe and effective amount of the vaccine of claim 17.

19. A method for enhancing production of a viral protein in Drosophila which comprises:

(a) transfecting a Drosophila cell with a viral gene expression unit, a rev gene expression unit, and a selectable marker which is on one or more DNA vectors;

(b) culturing said cell in a suitable medium; and

(c) collecting said protein.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/06838

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C12P 21/06; C12N 5/00, 1/22, 15/00; A61K 39/00, 38/12; C07H 15/12,
US CL: 435/69.1, 240.1, 240.2, 252.3, 320.1; 424/88, 89; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US	435/69.1, 240.1, 240.2, 252.3, 320.1; 424/88, 89; 536/27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Databases: Dialog (411)
Automated Patent System (File US PAT 1971-1991)

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y,P	US, A, 5,008,373 (Kingsman et al.) 16 April 1991, see entire document.	1-19
X,P	Bio/Technology, Vol. 9, issued February 1991, Culp et al., "Regulated Expression Allows High Level Production and Secretion of HIV-1 gp 120 Envelope Glycoprotein in Drosophila Schneider Cells", pages 173-177, see entire document.	1-19

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17 December 1991	22 JAN 1992
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Gian Wang Gian Wang

International Application No. PCT/US91/06838

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	International Conference AIDS, Vol. 5, issued 4-9 June 1989, Johansen et al., "Stable expression and secretion of recombinant HIV-1 envelope protein in Drosophila Schneider Cells", P. 584, see meeting abstract.	1-19

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers__________, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

see attached sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	First International Conference on Gene Regulation, Oncogenesis, AIDS, issued 15-21 September 1989, Arthos et al., "Interaction of the HIV Envelope with Human CD4 Receptor", see abstract.	1-19